

Genetic differentiation in the populations of red piranha, *Pygocentrus nattereri* Kner (1860) (Characiformes: Serrasalminae), from the river basins of northeastern Brazil

L. A. Luz^{a*}, L. L. Reis^a, I. Sampaio^b, M. C. Barros^{a,c} and E. Fraga^{a,c}

^aPrograma de Mestrado em Ciência Animal – CMCA, Centro de Ciências Agrárias – CCA, Universidade Estadual do Maranhão – UEMA, Tirirical, s/n, CEP 65058-250, Cidade Operária, São Luís, MA, Brazil

^bDepartamento de Química e Biologia, Instituto de Estudos Costeiros, Universidade Federal do Pará – UFPA, Alameda Leandro Ribeiro, s/n, CEP 68600-000, Aldeia, Bragança, PA, Brazil

^cLaboratório de Genética e Biologia Molecular, Departamento de Química e Biologia, Universidade Estadual do Maranhão – UEMA, Praça Duque de Caxias, s/n, CEP 65604-380, Morro do Alecrim, Caxias, MA, Brazil

*e-mail: lucianaluz_alves@hotmail.com

Received: January 17, 2014 – Accepted: July 18, 2014 – Distributed: November 30, 2015

(With 4 figures)

Abstract

The red piranha, *Pygocentrus nattereri*, is an important resource for artisanal and commercial fisheries. The present study determines the genetic differentiation among *P. nattereri* populations from the northeastern Brazilian state of Maranhão. The DNA was isolated using a standard phenol-chloroform protocol and the Control Region was amplified by PCR. The PCR products were sequenced using the dideoxyterminal method. A sequence of 1039 bps was obtained from the Control Region of 60 specimens, which presented 33 polymorphic sites, 41 haplotypes, $h=0.978$ and $\pi=0.009$. The neutrality tests (D and F_s) were significant ($P < 0.05$) for most of the populations analyzed. The AMOVA indicated that most of the molecular variation (72%) arises between groups. The fixation index was highly significant ($F_{ST} = 0.707$, $P < 0.00001$). The phylogenetic analyses indicated that the specimens represented a monophyletic group. Genetic distances between populations varied from 0.8% to 1.9%, and were $<0.5\%$ within populations. The degree of genetic differentiation found among the stocks of *P. nattereri* indicates the need for the development of independent management plans for the different river basins in order to preserve the genetic variability of their populations.

Keywords: DNA mitochondrial, control region, variability, management, conservation.

Diferenciação genética em populações da piranha vermelha, *Pygocentrus nattereri* Kner (1860) (Characiformes: Serrasalminae), em bacias hidrográficas do nordeste do Brasil

Resumo

A piranha vermelha, *Pygocentrus nattereri*, é um recurso importante para pesca artesanal e comercial. O presente estudo determinou a diferenciação genética entre populações de *P. nattereri* no nordeste do estado brasileiro do Maranhão. O DNA foi isolado utilizando o protocolo de Fenol-clorofórmio e a Região Controle foi amplificada por PCR. Os produtos da PCR foram sequenciados usando o método didesoxiterminal. Uma sequência de 1039 pbs foi obtida da Região Controle de 60 espécimes, que apresentaram 33 sítios polimórficos, 41 haplótipos, $h=0.978$ e $\pi=0.009$. Os testes de neutralidade (D and F_s) foram significativos ($P < 0.05$) para a maioria das populações analisadas. A AMOVA indicou que a maior parte da variação molecular (72%) surge entre os grupos. O índice de fixação foi altamente significativo ($F_{ST} = 0.707$, $P < 0.00001$). As análises filogenéticas indicaram que os espécimes representam um grupo monofilético. Distâncias genéticas entre as populações variaram de 0.8% a 1.9%, e de $<0.5\%$ dentro das populações. O grau de diferenciação genética encontrada entre os estoques de *P. nattereri* indicam a necessidade para o desenvolvimento de planos de manejo independentes para as diferentes bacias hidrográficas, a fim de preservar a variabilidade genética dessas populações.

Palavras-chave: DNA mitocondrial, região controle, variabilidade, manejo, conservação.

1. Introduction

Piranhas are Neotropical freshwater fishes, member of the order Characiformes a group of some ten families, 240 genera, and 1460 species found predominantly in the New World – from the southwestern United States and Central America to South America – as well as Africa that present a range of predatory behaviors (Santos et al., 2004; Calcagnotto et al., 2005), specifically the subfamily Serrasalminae, which includes approximately 80 species in 15 genera (Santana et al., 2011). Serrasalmines are present in all the river basins of South America (Oyakawa et al., 2006).

The red piranha, *Pygocentrus nattereri* Kner (1860), is characterized by its oval-shaped body, convex profile, medium size (up to 25 cm), and predominantly dark grayish-silvery coloration, which becomes reddish in the region of the pectoral, ventral, and anal fins. The teeth are resistant, sharp, strong, and dilacerating, which reflects the voracious carnivorous feeding behavior of these fishes (Soares, 2005; Santos et al., 2006). The species is found in a number of different river basins in South America (Queiroz et al., 2010). The ample distribution of the species, its relative abundance, and meaty body contribute to its use as a food by human populations, primarily for soups and sashimi (Barros et al., 2010).

This species is widely distributed in the Brazilian state of Maranhão, which represents an area of transition between the semi-arid region of northeastern Brazil to the east and the exuberant Amazon rainforest to the west, and a complex hydrographic system dominated by perennial rivers such as the Parnaíba, Itapecuru, Mearim, and Pindaré. The Pindaré, Mearim, and Itapecuru form a group of basins typical of the region, with similar topography, and all three discharge into the Gulf of Maranhão, the Mearim/Pindaré into São Marcos Bay, and the Itapecuru in São José Bay. The lower stretches of the Itapecuru and Mearim are characterized by a distinct landscape of meanders and lakes, and the Mearim connects to the Pindaré, which is its principal tributary (Teixeira and Souza-Filho, 2009; Piorski, 2010). The geomorphological evolution of the Gulf of Maranhão reflects the fluctuations in sea level that occurred during the Pliocene and Pleistocene, which culminated in the present-day configuration of estuaries in central Maranhão (Ab'Saber, 1960).

The successful exploitation of the fishery resources available in a river basin depends not only on the understanding of the life cycles of the local species, but also the genetic structure of their populations. Molecular techniques, including sequences of mitochondrial DNA (mtDNA) have proven especially effective for the evaluation of the genetic diversity of a wide range of fish taxa, and the Control Region has been used for the analysis of the population structure of a large number of species (Santos et al., 2004; Calcagnotto et al., 2005; Fraga et al., 2007; Cheng et al., 2012; Borba et al., 2013).

The taxonomy and systematics of the serrasalmines are subject to a great deal of controversy, and there is considerable disagreement on the position of the subfamily

in the Characiformes (Santana et al., 2011). A number of studies have raised questions with regard to the phylogenetic relationships among the species of the genus *Pygocentrus* (Freeman et al., 2007; Hubert et al., 2007; Orti et al., 2008). Analyzing the phylogeography of *P. nattereri* based on sequences of the Control Region, Torrico (2004) found evidence of population structuring in the specimens from the Madeira River, which formed a distinct group from those collected in the Solimões and Ucayali basins.

Despite its economic and nutritional importance, few data are available on the genetic structure of the *P. nattereri* populations of the river basins of the Brazilian Northeast, and no information whatsoever exists for the state of Maranhão. As the exploitation of fishery stocks without any prior knowledge of their genetic variability may hamper management initiatives and sustainability, the present study investigated the genetic differentiation of the *P. nattereri* populations of the principal river basins of Maranhão, in northeastern Brazil, based on the sequencing of the mitochondrial Control Region.

2. Material and Methods

2.1. Collection and extraction of DNA

A total of 60 *Pygocentrus nattereri* specimens were collected from the basins of the Itapecuru (municipality of Itapecuru-Mirim), Mearim (municipality of Pedreiras), Pindaré (municipality of Pindaré-Mirim), and Parnaíba (municipality of Coelho Neto) rivers in the Brazilian state of Maranhão (Figure 1), with each basin being represented by 15 specimens. The specimens were identified based on the specific literature (Britski et al., 1999; Santos et al., 2004, 2006; Soares, 2005) and confirmed by specialists from the Museum of Zoology at São Paulo University, MZUSP (vouchers - 104549/104550). All specimens were euthanized by immersion in ice water (Ashley, 2007) and deposited in the collection of the Genetics and molecular Biology Laboratory of Maranhão State University in Caxias. Samples of muscle tissue were extracted from each specimen and conserved in 70% ethanol. The DNA was extracted using the standard phenol-chloroform protocol (Sambrook and Russel, 2001). The collection of specimens was authorized by IBAMA, the Brazilian Federal Environment Institute (license number 02012.004159/2006).

2.2. PCR and sequencing

The mitochondrial Control Region was isolated and amplified using the Polymerase Chain Reaction (PCR) based on the primers D-LoopL1 '5 CTAAGTCCCAAAGCTAGGTATTC3' and D-LoopH1 '5 TGTTTATCACTGCTGRRITCCCT 3' (Santa Brígida et al., 2007). The PCR was run in a final volume of 25 µl composed of 4 µl of DNTPs (1.25 M), 2.5 µl of buffer solution (10X), 0.5 µl of MgCl₂ solution (50 mM), 1 µl of DNA (250 ng/µl), 0.25 µl of each primer (200 ng/µl), 0.2 µl of the Taq polymerase enzyme (5U/µl), and 16.3 µl of purified water.

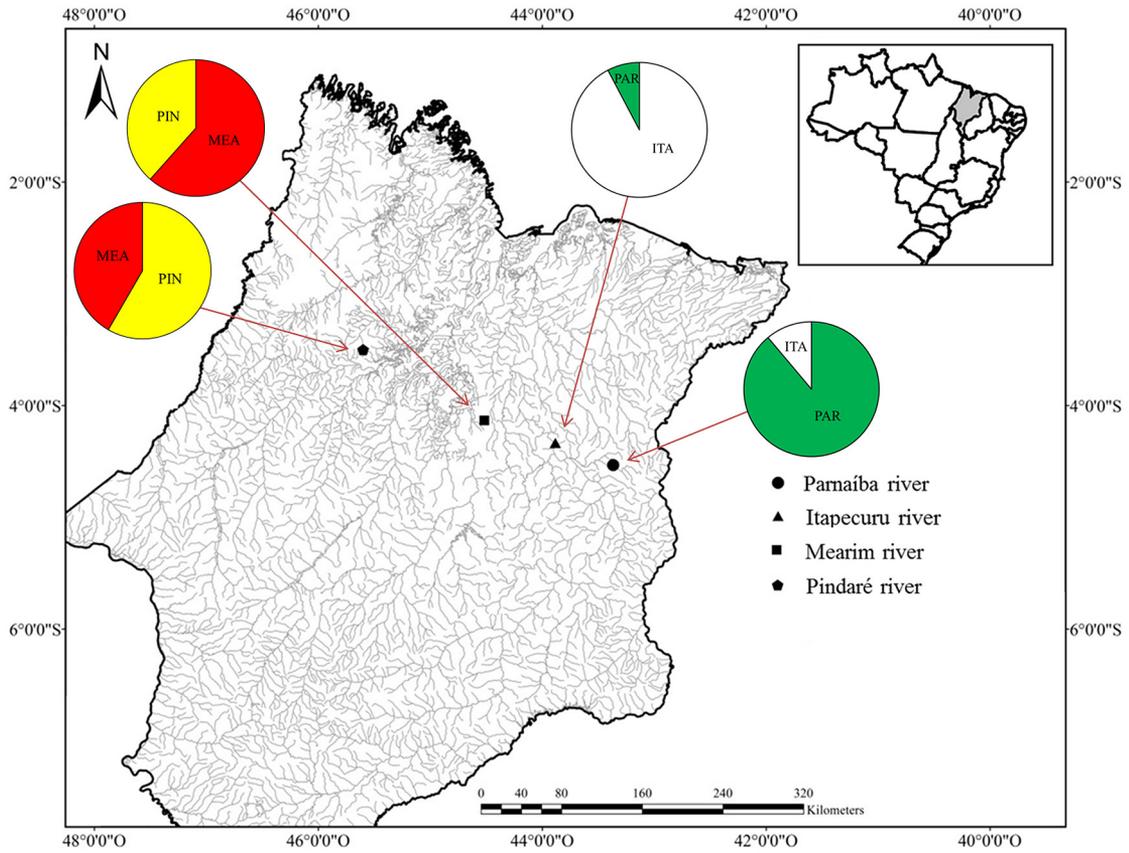


Figure 1. Location of the river basins in the Brazilian state of Maranhão in which the specimens of *Pygocentrus nattereri* were collected for analysis in the present study. The circles represent the proportion of the haplotypes shared between basins. PAR = Parnaíba, ITA = Itapecuru, MEA = Mearim, and PIN = Pindaré.

The amplification protocol consisted of 3 min at 94°C, followed by 35 cycles of 30 s at 94°C, 1 min at 50°C, 2 min at 72°C, and a final extension of 7 min at 72°C. The PCR products were visualized in a 1% agarose minigel with ethidium bromide and purified with ExoSAP-IT according to the manufacturer's protocol. The sequencing reaction was based on the Sanger et al. (1977) technique, using an ABI Prism TM Dye Terminator v 3.1 Cycle Sequencing Reading Reaction kit (Applied Biosystems). The samples were precipitated in EDTA/sodium acetate/ethanol and analyzed in an automatic DNA sequencer (ABI 3500/Life Technologies).

2.3. Phylogenetic and population analyses

The sequences were edited in Bioedit 7.0 (Hall, 1999) and aligned using the Clustal W 1.4 tool (Thompson et al., 1994). Two sequences of the Control Region of *Pygocentrus nattereri* deposited in the Genbank (AF283953 - Orti et al., 2008 and AP012000 - Nakatani et al., 2011) were used as a reference for the alignment. The most adequate evolutionary model for the dataset was identified by JmodelTest 2 (Darriba et al., 2012). Tamura's (1992) T92+G+I model was selected for the construction of the trees using the Akaike Information Criterion (AIC), with the parameters

being derived from Maximum Likelihood (ML) and Neighbor-Joining (NJ) approaches. The phylogenetic analyses Maximum Likelihood (ML) and Neighbor-Joining (NJ) were run in PHYML 3.0 (Guindon et al., 2010). The significance of the groupings produced by these analyses was tested by bootstrap analysis with 1000 pseudoreplicates (Felsenstein, 1985). Sequences of *Metynnys hypsauchen* (D-Loop AF283957 - Orti et al., 2008) and *Metynnys* sp. (D-Loop AF283956 - Orti et al., 2008) were used as the outgroup.

The haplotype and nucleotide diversity indices were obtained from DNASP 5.1 (Librado and Rozas, 2009), and the haplotype network was generated by Haploviewer software (Salzburger et al., 2011), using the Maximum Likelihood, in PHYML 3.0 (Guindon et al., 2010). Evidence of selective neutrality in the populations was gleaned from the D (Tajima, 1989) and F_s tests (Fu, 1997). The possible existence of differentiated populations and the significance of inter- and intra-population variability were verified using an Analysis of Molecular Variance (AMOVA). Five different AMOVAs were run, four of which included only the populations from Maranhão, and a fifth that included 30 sequences of the Control Region of *P. nattereri* obtained from Genbank, representing populations

from the basin of the Amazon River (Amazonas, Brazil) – KC132013/ KC131975-79 (Thompson et al., 2014), DQ384773-75 and DQ384781-83 (Hubert et al., 2007), AP012000 (Nakatani et al., 2011) and AF283953 (Orti et al., 2008), the Madeira River (Amazonas, Brazil) – DQ384764-72 (Hubert et al., 2007), the Paraná River (Paraná, Brazil) – DQ384776-80 (Hubert et al., 2007), and the São Francisco River (Bahia, Brazil) – DQ384784-85 (Hubert et al., 2007). The fixation index (F_{ST}) and its significance were obtained from 1023 random permutations. The neutrality tests of AMOVA and fixation indices were obtained in the Arlequin 3.5 program (Excoffier and Lischer, 2010). Possible population groups were identified in BAPS 6.0 using the Bayesian grouping analysis described by Corander et al. (2013). The nucleotide divergence indices were determined by the uncorrected p distances and the corrected Tamura and Nei (1993) parameters in Mega 6.0 (Tamura et al., 2013) considering the groups generated in the BAPS analysis.

3. Results

The amplification of the Control Region of the 60 *Pygocentrus nattereri* specimens generated a sequence of 1039 base pairs. The mean nucleotide composition of these sequences was 29.8% Thymine, 23.4% Cytosine, 30.6% Adenine, and 16.2% Guanine. A total of 33 polymorphic sites and 41 haplotypes were identified, with a general haplotype diversity of 0.978 and nucleotide diversity of 0.009. High levels of haplotype diversity were found in each of the populations analyzed (Table 1).

Overall, 32 of the 41 haplotypes identified in the analyses were unique and exclusive. Of these, seven (H2–H5 and H7–H9) were from the Parnaíba basin, 12 (H10–H21) were from the Itapecuru, eight (H23, H24, H28–H32, and H34) were from the Mearim, and the remaining five (H36–H39 and H41) were from the Pindaré. The most common haplotype was H6, which was recorded six times and was found in both the Parnaíba and Itapecuru basins. The second most common haplotype was H1, which was exclusive to the Parnaíba, with five records. The third most common haplotype was H26, with a total of four records from the Mearim and Pindaré basins, while the fourth most common was also found in these two basins, with a total of three records. Three other haplotypes –

H22, H27, and H33 – were also recorded from these two basins, with one record from each. The other haplotypes with two records – H35 and H40 – were exclusive to the Pindaré River (Figure 2).

The haplotype network (Figure 2) revealed a higher degree of similarity between the specimens from the Parnaíba and Itapecuru basins, even though one group of haplotypes from the Parnaíba is separated from the Itapecuru by three mutation events. The specimens from the Mearim and Pindaré basins were also highly similar, and were differentiated from the Parnaíba and Itapecuru populations by 12 mutations. These findings were further reinforced by the analyses of genetic distance (p distance, and Tamura and Nei), with distances of up to 1.9% between the Parnaíba population and that of Mearim-Pindaré, and 1.4% between Itapecuru, Mearim-Pindaré.

The results of the neutrality tests – Tajima's D and Fu's F_S – were significant ($P < 0.05$) for the majority of the populations (except for the Parnaíba River – $F_S = -0.976$, $P = 0.283$) when analyzed together or separately (Table 1). This indicates that the hypothesis of neutral polymorphism can be rejected, and suggests that the populations passed through a recent process of expansion.

The patterns of genetic variability found within and between populations in the Analysis of Molecular Variance

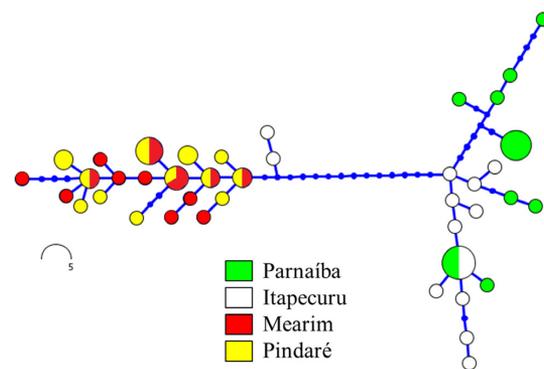


Figure 2. Haplotype network based on a maximum likelihood analysis of the sequences of the mitochondrial Control Region. Each haplotype is represented by a circle (the size of which is proportional to its total frequency in the populations) with the different colors representing each of the study populations.

Table 1. Levels of genetic diversity and the results of the neutrality tests for the populations of *Pygocentrus nattereri* from the Brazilian state of Maranhão analyzed in the present study, based on sequences of the mitochondrial Control Region.

Population	N	NH	S	Index of molecular diversity		Neutrality tests		
				h	π	D	F_S	P
Parnaíba river	15	09	14	0.876	0.005	0.840	-0.976	0.283
Itapecuru river	15	13	18	0.971	0.005	-0.022	-6.072	0.001
Mearim river	15	13	09	0.981	0.002	-0.008	-10.55	0.000
Pindaré river	15	12	09	0.971	0.002	-0.188	-8.503	0.000
Total	60	41	33	0.978	0.009	0.155	-6.527	0.071

N = sample size; NH = number of haplotypes, S = polymorphic sites; h = haplotype diversity, π = nucleotide diversity, D = Tajima's index, F_S = Fu's index, and P = significance of the F_S value. Significance level ($P < 0.05$).

(AMOVA) were based on the three principal clusters, i.e., Parnaíba, Itapecuru, and Mearim/Pindaré. The AMOVA indicated that 72% of the molecular variation was found among groups, with only 29% being found within populations. The F_{ST} value was 0.707, and highly significant ($p < 0.00001$), indicating the existence of genetic structuring among the study populations (Table 2). When the populations were considered as a single group, the differentiation among populations was 68%, and the F_{ST} value (0.667) was still highly significant ($p < 0.00001$). Three other simulations were run on the Maranhão populations (Parnaíba vs. Mearim-Pindaré, Itapecuru vs. Mearim-Pindaré and Parnaíba, Itapecuru vs. Mearim-Pindaré), and the results were always $F_{ST} < 0.70$ with $p < 0.00001$.

The results of the AMOVA that included the 30 *P. nattereri* sequences obtained from Genbank, and tested the hierarchy of three groups – North (Amazon and Madeira basins), South (Paraná River), and Northeast (Parnaíba, Itapecuru, Mearim, Pindaré and São Francisco basins) – found that most (46%) of the molecular variation is found among populations of the same region. The F_{ST} value was 0.716, with a highly significant $p < 0.00001$, indicating the existence of a high degree of genetic structuring among populations (Table 2).

The phylogenetic analyses were based on two different approaches – ML and NJ – in order to estimate the most likely topology of the evolutionary history of the *P. nattereri* populations in the study area. Both methods produced highly similar topologies, grouping the specimens with 100% bootstrap values. The different phylogenetic approaches (Figure 3) also revealed the existence of three distinct groups formed by the specimens from the Parnaíba basin (ML and NJ with 85% bootstrap values), samples from the Parnaíba and Itapecuru rivers (ML = 69%, NJ = 84%), and specimens from the Itapecuru, Mearim, and Pindaré basins (ML = 92%, NJ = 95%).

The population groupings generated by the BAPS analysis, based on Bayesian inference, revealed the existence of three groups or clusters (Parnaíba, Itapecuru, and Mearim-Pindaré), with the greatest distance being found between the Parnaíba and Mearim-Pindaré groups, with a divergence of 1.9% (Figure 4, Table 3). The smallest

divergence (0.8%) was found between the Parnaíba and Itapecuru groups, while within-population differentiation was invariably less than 0.5% (Table 3).

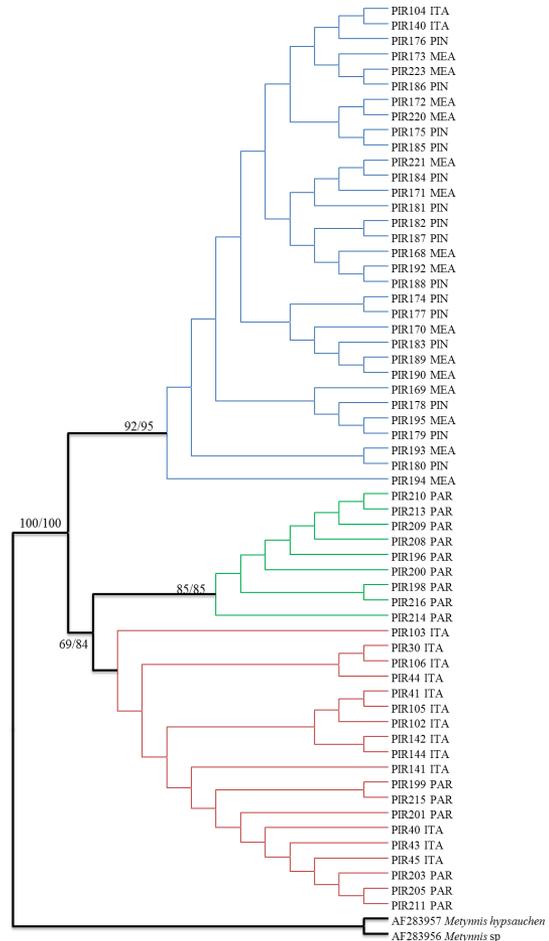


Figure 3. Phylogenetic tree derived from 1000 bootstrap replicates based on the Tamura (1992) model. The numbers above the branches represent the bootstrap values for the Maximum Likelihood/Neighbor-Joining approaches, respectively. PAR = Parnaíba, ITA = Itapecuru, MEA = Mearim, and PIN = Pindaré.

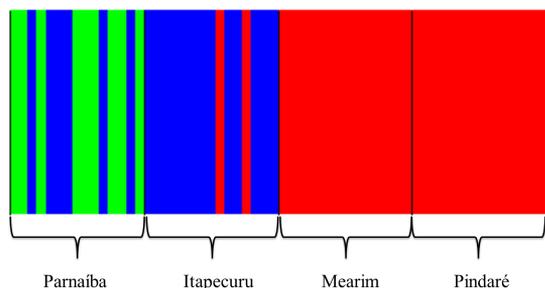
Table 2. Results of the AMOVA for the populations of *Pygocentrus nattereri* obtained for the sequences of the mitochondrial Control Region.

Type of variation	Component of the variation	% of the total variation	F_{ST}	P
Clusters: Parnaíba, Itapecuru and Mearim-Pindaré				
Between groups	4.932	72	0.707	<0.00001
Between populations of the same groups	-0.072	-1		
Within populations	2.014	29		
Groups: North, South and Northeast				
Between groups	129.617	26	0.716	<0.00001
Between populations of the same groups	189.116	46		
Within populations	154.667	28		

Significance level ($P < 0.05$).

Table 3. Mean nucleotide divergence (percentage) among *Pygocentrus nattereri* populations from the Brazilian state of Maranhão based on p (below the diagonal) and Tamura and Nei (above the diagonal) distances.

Population	% Inter-population divergence			% Intra-population divergence
	Parnaíba	Itapecuru	Mearim-Pindaré	
Parnaíba		0.8	1.9	0.3
Itapecuru	0.8		1.5	0.2
Mearim-Pindaré	1.9	1.4		0.3

**Figure 4.** *A priori* estimate of the probable groups of populations produced by the BAPS (Bayesian Analysis of Population Structure v 6.0) program, indicating a total of three groups.

4. Discussion

In the present study, the estimates of polymorphism in *Pygocentrus nattereri* indicated high levels of genetic variability in the river basins analyzed. Haplotype diversity was high considering the populations either as a group ($h = 0.978$) or individually. The genetic variability of a population may determine its capacity to adapt to changes in the environment, reproductive success and growth rates and, ultimately, its chances of survival over the long term (Frankham et al., 2002).

The haplotype network indicated a greater genetic similarity between the specimens from the Mearim and Pindaré basins, emphasized by the largest number of shared haplotypes (five). This marked similarity between the basins was further emphasized by the results of the BAPS analysis, with the two groups being included in a single cluster. The genetic proximity between the specimens from these two basins may be a result of their geomorphological similarities, and not least because the Pindaré is the principal tributary of the Mearim (Teixeira and Souza-Filho, 2009; Piorski, 2010).

The neutrality tests – D (Tajima, 1989) and F_s (Fu, 1997) – were significant ($P < 0.05$) for most populations, indicating that they may have passed through a recent process of expansion. This conclusion is supported by the large number of unique haplotypes found in these populations, given that rapid population growth favors the retention of new mutations (Avise et al., 1984).

The results of the AMOVA indicated that the majority (72%) of the genetic variation was related to differences among the groups. The highly significant F_{ST} value (0.707; $p < 0.00001$) indicated that the *P. nattereri* populations are

genetically structured. A similar pattern was observed in the populations from the Northern, Northeastern and Southern regions ($F_{ST} = 0.716$; $p < 0.00001$). Defining genetic variation at the population level is essential for the understanding of the distribution of this diversity within the species. When this variation is continuous, any given area within its geographic range should be representative of the species, whereas genetic structuring indicates the need to consider each subpopulation as a separate unit (Terencio, 2009).

The phylogenetic analyses based on the different analytical approaches (ML and NJ) and with bootstrap values of 100%, indicated the existence of three distinct groups (Parnaíba, Itapecuru-Parnaíba, and Mearim-Pindaré). The concept of evolutionary units refers to the definition of a population or group of populations differentiated genetically, morphologically or ecologically from neighboring conspecific populations, reflecting a historical process of geographic isolation on some level. As they present distinct characteristics, these groups should be treated as independent units for conservation purposes (Terencio, 2009). Torrico (2004) reported that the *P. nattereri* specimens from the upper Madeira, Solimões, and Ucayali Rivers grouped together with a 100% bootstrap value. Ortí et al. (2008) also confirmed the monophyly of the serrasalmine based on an analysis of the mitochondrial Control Region. Similar results were obtained using this marker for the serrasalmine genera *Serrasalmus* (Hubert et al., 2007) and *Pygocentrus* (Freeman et al., 2007).

The highest genetic divergence indices between populations were found between Parnaíba and Mearim/Pindaré (1.9%). Low levels of divergence (<0.5%) were found within the populations analyzed. Hubert et al. (2007) recorded genetic distances of 1.2% and 3.0% between serrasalmine species, and distances of 0.1% to 0.8% between populations of the same species. In a phylogeographic study of *P. nattereri*, Torrico (2004) recorded divergence values of between 0.29% and 1.12% for specimens from the Madeira, Ucayali, and Solimões river basins. Analyzing the Control Region, Ortí et al. (2008) recorded genetic distances of 0.017% to 0.256% between serrasalmine species.

The definition of biological parameters is extremely important for the development of adequate procedures for the management of natural resources, such as fishery stocks. Studies of genetic variability, principally those based on polymorphisms of mitochondrial DNA, provide a strong scientific baseline for the development of conservation and management programs (Batista, 2010).

In the present study, the results of the analysis of the Control Region provided important insights into the genetic variability of the *Pygocentrus nattereri* populations of the study area, and in particular, their structuring among the different river basins revealed consistently by the different analytical approaches, all of which supported the presence of three distinct groups (Parnaíba, Itapecuru, and Mearim-Pindaré). These findings will be important for the development of effective conservation and management policies, which should treat each basin separately, given the genetic differences found between them.

Acknowledgements

We are grateful to the Bank of Northeastern Brazil (BNB) and the Maranhão State Scientific and Technological Development Foundation (FAPEMA) for financial support. We would also like to thank Luís Fernando Silva Rodrigues, a post-doctoral fellow at the Institute of Coastal Studies at UFPA/Bragança campus for conducting the analyses.

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